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Violent cavitation from optically configured microbubble pairs

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Contrast agent microbubble cavitation has received increased attention in recent years, since it has been recognised as significantly enhancing the bio-effects of ultrasound mediated intra-cellular drug delivery, the technique better known as sonoporation. However, whilst the empirical credentials underscoring the potential of sonoporation to usefully load the full gamut of biological cells and tissues continues to grow, there remains a distinct lack of clarity in relation to the underlying mechanism of action. Here, we have adopted the viewpoint that this aspect will only become elucidated by imaging the interaction of individual ultrasound stimulated microbubbles with nearby cells, and corroborating subsequent damage or disruption to that specific modes of bubble activity. Previous studies that have used time lapse photomicrography to image free microbubbles have demonstrated their vigorous interaction with pressure pulses, especially at mechanical indices greater than circa 0.5. For the purposes of the present paper, we extend that approach and use corroborative cell imaging by both electron- and force microscopies to illuminate an unusual mode for membrane disruption from violent microbubble cavitation.

1 Introduction

Microbubble contrast agents were originally developed to enhance echogenicity in diagnostic sonography. However, their somewhat unique acoustic response and facility to transduct energy into spatially focussed regimes, together with the clinical demand for non-invasive adjuncts and options to conventional therapy, have seen interest in exploring the therapeutic potential of microbubbbles grow steadily within this past decade [1]. For the purposes of the present study, we examined the ultrasonically stimulated response of many such microbubbles by assessing their specific damage pattern on cell membranes. One objective was to improve our understanding of their statistical behaviour and to feed this information into realistic computational models so that protocols for sonoporation might be more easily optimised. Bulk injected bubble populations were insonated using a burst of 1MHz ultrasound at a nominal peak pressure of between 0.5-6.5MPa. Subsequently, corroborative bubble dynamics were recorded using high speed micro-photography and any resultant damage induced at the target surface was also observed and characterised using a range of high resolution imaging technologies including atomic force microscopy (AFM) and scanning electron microscopy (SEM).

2 Apparatus & Method

The salient points of our apparatus are outlined below. The main fluid chamber consists of a 290 x 290 x 90mm perspex tank that is filled with degassed water. On the front face of the tank, a 37 x 20 x 50 mm perspex compartment is attached which contains an angled acetate window, affording good acoustic impedance matching with a main tank and sealing off the leading edge of the compartment. Three glass fibre bundles are embedded into the compartment, with the output end below the acetate window, to which the flashlights are coupled for the purposes of illumination. Two bevelled Perspex struts provide a mounting for a 10.5x22 mm oblong plastic Thermanox® coverslip (Nalge Nunc International), such that it is maintained vertically, and the top edge of the substrate is aligned to the top edge of the compartment. A glass microscope coverslide is placed across the top of the compartment on completing sample preparation and the chamber positioned beneath the microscope objective lens.

Ultrasound (US) was generated with a 1.0 MHz focussed piezoceramic transducer (Sonic Concepts INC, model no. H-101 S/N -27). The insonation protocol typically employed a single US burst constituted of 20 pulses. The transducer is driven by a signal generator (Stanford Research System, model no. DS345, 30MHz synthesized function generator) operating in the sinusoidal mode. This signal is amplified with a gain of up to 200 by a radio-frequency power amplifier (Amplifier Research, model no. 150A100D 150 Watts 10kHz – 100 MHz). A TTL pulse from the signal generator, generated in coincidence with the sinusoidal pulse was also used to trigger a high speed camera. The average peak-to-peak pressure at the focus of the ultrasonic field is determined using a needle hydrophone (Precision Acoustics Ltd, serial no. 839), as 6.5±1.3 MPa. The delay between the trigger pulse from the signal generator and registration of the ultrasound burst at the hydrophone was typically 45μs and thus acquisition of the high speed camera data was post-triggered by this interval.

The procedure commences with injection of a small volume of microbubble solution into the partitioned volume of the US chamber. A small volume is preferred as surplus microbubbles only serve to attenuate the incident US and distorts the response of the target microbubble; 1.5μL is typically used. The microbubbles rise under the action of buoyancy forces to the surface of the containing medium. A cleaned microscope coverslide is then placed over the microbubble cloud and the chamber positioned below the objective lens. The objective lens is set to its maximum working distance of 230μm. The top edge of the vertically aligned substrate that is within the US focal region is then located, and any microbubbles in close proximity become candidates for local insonation, although 'sticking' and 'clumping' of particles restricts the number actually available.

3 Results

Chemical fixing (4% gluteraldehyde solution for 20 mins) of both the control and insonated cell monolayer samples, high resolution AFM analysis was undertaken using standard silicon tips for 'tapping' mode imaging in air. On the control samples that had not been subjected to insonation it was found that the surface topography [of DU145 prostate cancer cells] was dominated by microvilli and also a population of shallow (70-90nm) pits, possibly representing areas of exocytosis. In contrast, insonated samples exhibited an almost complete loss of microvilli. Further, distinct topographical features were occasionally

observed on the regions on the periphery of the CZs. Unlike the jet formed sonopores that we have reported previously [2] which typically exhibit a distinct ring of raised material around the pore site, pores observed on the periphery of CZs were simply wider and deeper (>100nm) versions of regular pores found on native control samples.

Characterization of the typical topographical and staining outcomes were ascertained by undertaking preliminary insonations on cell monolayers. Inspection of insonated monolayers showed that large swathes of cells had been removed to create circular and, more generally, elliptical regions of exposed substrate. Such exposed regions are termed 'clearance zones' (CZs). One such example (*viz* Fig.1) also demonstrated that those cells on the periphery of a CZ exhibit calcein [which was present in the mother liquor] uptake and must thus have experienced some form of permeabilisation. Implementing dual staining with propidium iodide as a [red] viability stain, it also became clear that a few (red) cells have become non-viable in this process.

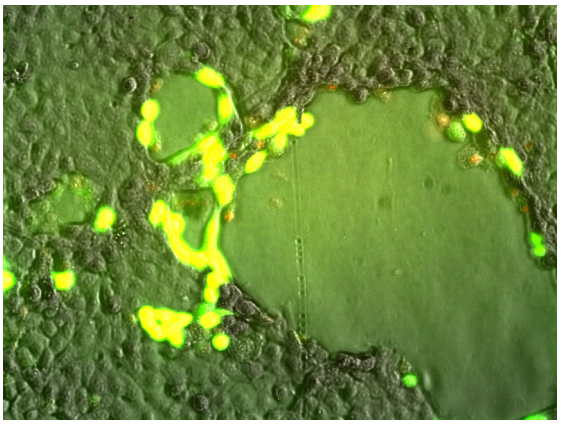

FIG. 1. Formation of clearance zones and cell uptake during insonation. Fluorescence microscopy indicates that molecular uptake of calcein (green) has occurred, predominantly in those cells on the periphery of the clearance zones as well as a few interspersed PI stained cells that have been irreparably sonoporated.

Occasionally, insonated samples showed evidence for multiple puncture events in very close proximity. Here (viz Fig. 2), sonopore sizes are typically much smaller (sub-micron) than the jet induced entities (circa 10 microns) that we have observed previously and their formation could arise as a result of several distinctly different microbubble events, as will be discussed presently.

3 Discussion

How is it that multiple sonopores can arise so close together? This question is especially pertinent in the light of previous results that suggest that micro-jetting [2] or bubble fragmentation is the critical process leading to ballistic penetration of cell membranes. Interestingly however, parallel studies of insonated monolayers that were undertaken with SEM may provide some further clues to this occurrence: indeed we have observed some compelling circumstantial evidence for microbubble mediated

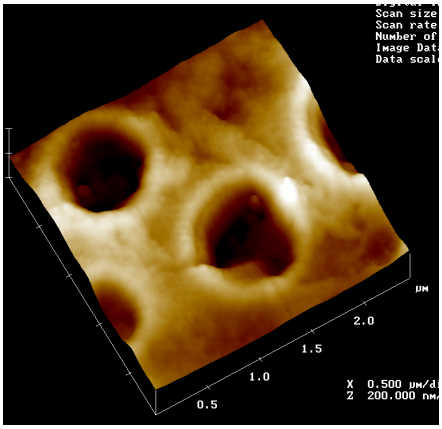

FIG 2. Force microscopy on an insonated cell monolayer showing micrometer sized 'sonopores'. Notably, the topography on control samples that had not been insonated did not exhibit such features and thus we infer that they arise as a result of interaction with microbubbles.

permeabilisation that suggests that the role for 'sonic cracking' of microbubbles may have been previously underestimated. Here, the huge benefit of SEM imaging was that it allows fast examination over a larger area of target material. We have observed (viz Fig. 3) that a ruptured Optison bubble remained at the scene of membrane disruption.

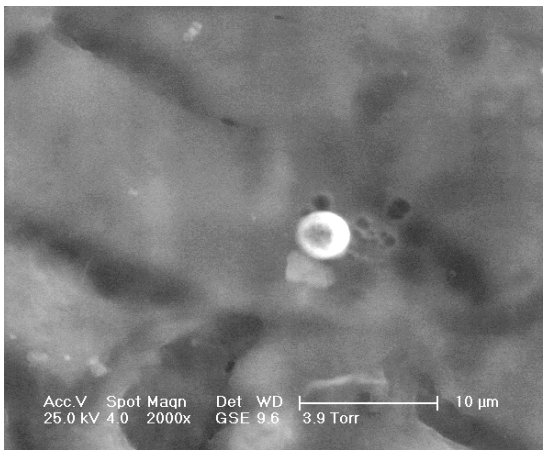

FIG. 3. A ruptured Optison bubble, with evident shell discontinuity, is seen resting on a cell membrane exhibiting multiple local sites of disruption. This observation supports the hypothesis that a sonically cracking microbubble can continually 'bounce' against the membrance during gas release and stretch the membrane beyond its critical yielding threshold.

Regarding the general observation, we suggest that the formation of a clearance zones, and the formation of individual sonopores via a bubble derived microjetting process, are aspects of the same phenomena but occurring at different length scales. We have shown previously that membrane breaching due to microjetting from a cavitating UCA results in the formation of sonopores, the diameter of which appears to scale with the ultrasound amplitude for a given initial bubble displacement. Similarly, the pattern of molecular uptake observed around the periphery of CZ's

can arise through the nucleation and development of a free bubble. Our principal reasoning for this conjecture comes from the fact that we have observed the formation of CZs on insonated cell monolayers *without* the presence of UCA. The detachment of cells from a substrate exposed to lithotripter generated shock waves has been reported before and been attributed to the natural nucleation of millimetre sized cavities from the ambient medium. As these cavities evolve in the vicinity of the rigid boundary, then the imposition of that boundary to the fluid dynamics acts to give rise to jet formation in a very natural way. A radial flow is subsequently created when the re-entrant jet impinges with the solid cover-slip, causing cells to become removed from the CZ regions simply because they are subjected to a radial shear force that is sufficient to overcome their bonds with the underlying substrate. Moreover, we observe that the cells to the edge of a CZ appear compressed in the direction moving radially away from the geometric centre of the CZ suggesting that the radial flow is forces the cell together. Moreover, the reason why peripheral cells experience prevalent molecular uptake may then be because they are subjected to a shear flow that is sufficient to disrupt the membrane. Certainly the development of pore features that are much larger than those found on control cells that had not been exposed to ultrasound supports this suggestion.

We observed recovery times of circa 10s [data not shown] is consistent with recent work employing a voltage patch clamp technique to measure membrane disruption of xenopus oocytes in response to ultrasound exposure, again in the presence of Optison. It was reported that the transmembrane current increased, due to decreasing membrane resistance attributed to pore formation, and that the size of the current escalation increased with Optison concentration. Furthermore, the current regained it pre-exposure value in a matter of seconds following insonation. We speculate that cells exposed to sub-lethal microjets recover the membrane by a vesicle patching response as suggested previously.

Ongoing work by us seeks to discriminate the exact mechanism of sonopore repair in terms of fundamental cellular activities.

4 Conclusions

In conclusion, we have investigated microbubble-cell interactions in response to insonation by microscopy imaging. We postulate that observations of pores, which are typically about twice the size of naturally occurring pores on the membrane, and which are found on cells left on the periphery of CZs, arise via a shear flow along the surface caused by cavitation of naturally occurring free bubbles. Similar observations with lithotripter shockwaves bolster this argument. On the other hand, ‘sonopores’ arising on cells well away from CZ’s, can be attributed to not only microjetting events arising from nearby UCA but also clearly from sonic cracking. The timescale for stanching of a non-lethal sonopore appears to be of the order of seconds, Further experiments are needed to determine the exact mechanism of cell membrane repair in this context and in particular, whether or not a vesicle patching response is the process at large.

Acknowledgments

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